

# Rapid and specific reactive oxygen species generation via NADPH oxidase activation during Fas-mediated apoptosis

Yoshihiro Suzuki<sup>a,\*</sup>, Yasushi Ono<sup>a</sup>, Yoshio Hirabayashi<sup>b</sup>

<sup>a</sup>Department of Microbiology, Nihon University School of Medicine, Oyaguchikami-machi, Itabashi-ku, Tokyo 173, Japan

<sup>b</sup>Laboratory for Cellular Glycobiology, Frontier Research Program, The Institute of Chemical and Physical Research (RIKEN), Saitama, Japan

Received 3 February 1998; revised version received 19 February 1998

**Abstract** To determine Fas-induced reactive oxygen species (ROS) generation in cells sensitive to Fas-mediated apoptosis, chemiluminescence, a strong indicator of ROS generation, was monitored after stimulation with agonistic anti-Fas. Fas ligation resulted in a rapid and sustained ROS generation. The generation reached a maximum within 5 min and was still observed at least 40 min after the stimulation. No significant ROS generation was observed under conditions where apoptosis was absent. Furthermore, an NADPH oxidase inhibitor, diphenylene iodonium, prevented Fas-mediated ROS generation, suggesting that Fas induces ROS generation via stimulation of the oxidase system.

© 1998 Federation of European Biochemical Societies.

**Key words:** Fas; Apoptosis; Reactive oxygen species; NADPH oxidase

## 1. Introduction

Fas/APO-1 (CD95) is a 36-kDa transmembrane type I receptor which belongs to the nerve growth factor/tumor necrosis factor receptor family [1]. Upon interaction with its agonistic antibody or its natural Fas ligand, Fas induces apoptosis in a number of cell lines including lymphocytes [2–4] and it seems to be particularly important in the homeostasis and development of lymphocytes (reviewed in [4]). However, despite many efforts to understand the mechanisms by which the Fas receptor transduces the death signal, the intracellular signaling cascade leading to apoptosis is poorly defined.

Several lines of evidence suggest the involvement of oxidative stress, a putative mediator of apoptosis [5], in Fas-mediated apoptosis. Various antioxidants such as thioredoxin, *N*-acetylcysteine (NAC), *N*-*t*-butyl-phenylnitrone, and glutathione (GSH) prevent Fas-mediated apoptosis in activated peripheral T cells, several T cell lines, and monocytes [6–10]. Furthermore, Fas-mediated apoptosis is modulated by intracellular GSH in these T cells [7,8]. Increasing the intracellular GSH content by the addition of the GSH precursor NAC decreases cellular sensitivity to Fas-mediated apoptosis, while depleting GSH by inhibiting its synthesis increases cellular sensitivity to apoptosis, without affecting surface Fas expression [8]. In addition, Fas ligation has been shown to decrease intracellular GSH content and to drive the cellular redox environments towards oxidizing redox status by stimulating the efflux of GSH [11]. These observations strongly suggest that stimulation via Fas may lead to oxidative stress by decreasing intracellular GSH, the major buffer of the cellular redox sta-

tus [12], and/or by increasing cellular reactive oxygen species (ROS). Consistent with this, it has been demonstrated that Fas ligation induces ROS generation in Jurkat T cells [9] and monocytes [10]. Furthermore, several lines of evidence suggest the significance of such ROS generation in Fas-mediated apoptosis, although the mechanisms underlying the ROS generation remain to be unclear [9,10]. By contrast, some investigators have demonstrated that Fas ligation does not enhance ROS generation, although Fas-mediated apoptosis is prevented by a range of antioxidants [7]. Most of these previous studies analyzed intracellular ROS generation by flow cytometric analysis using a fluorescent probe, dichlorofluorescein diacetate, on the basis of the specific reactivity of the fluorescent probe with H<sub>2</sub>O<sub>2</sub> [13]. However, subsequent studies revealed that the reactivity of the probe represents the occurrence of overall oxidative stress but not that of H<sub>2</sub>O<sub>2</sub> [14]. In addition, the rapid leakiness of the probe might cause the failure of ROS detection in some cases. Thus, examination by another method seems to be necessary to determine whether or not Fas induces ROS generation in Fas-sensitive cells.

To this aim, we attempted to utilize chemiluminescence (CL), a strong indicator of ROS generation that has been widely used in the detection of ROS from phagocytic cells [15,16]. We used luminol to amplify light production because it can react with a variety of ROS including O<sub>2</sub><sup>-</sup>, the hydroxyl radical (OH<sup>•</sup>), and singlet oxygen. Here we demonstrate that Fas ligation induces a rapid and specific ROS generation via NADPH oxidase activation during Fas-mediated apoptosis.

## 2. Materials and methods

### 2.1. Reagents and monoclonal antibodies (mAbs)

Luminol and catalase were obtained from Sigma (St. Louis, MO). Diphenylene iodonium chloride (DPI) was from Funakoshi Co. Ltd. (Tokyo, Japan). The agonistic anti-Fas mAb (clone CH-11) and the antagonistic anti-Fas mAb (clone ZB4) were purchased from MBL (Nagoya, Japan). The mAbs to human IgM, CD40, CD80, and CD81 were all obtained from Immunotech (Marseilles, France).

### 2.2. Cells

The human B lymphoma cell lines BJAB and Ramos and the human T-cell leukemic cell line Jurkat were cultured at 37°C in RPMI 1640 medium (Sigma) supplemented with 10% FCS (Hyclone), 100 U/ml penicillin, and 100 µg/ml of streptomycin in a humidified atmosphere containing 5% CO<sub>2</sub>/95% air.

### 2.3. Determination of cell viability

After incubating cells (1 × 10<sup>6</sup>/ml) with the agents tested for 18 h, the samples were added with an equal volume of 0.4% trypan blue and the dye-excluding cells were directly counted in a hemocytometer microscopically. In some experiments, cell viability was measured by MTT assay as previously described [17] except that a compound which releases a soluble formazan product (Dojin Laboratories, Kumamoto, Japan) was used. Briefly, cells in a 96-well tissue culture

\*Corresponding author. Fax: (81) (3) 5991-9865.

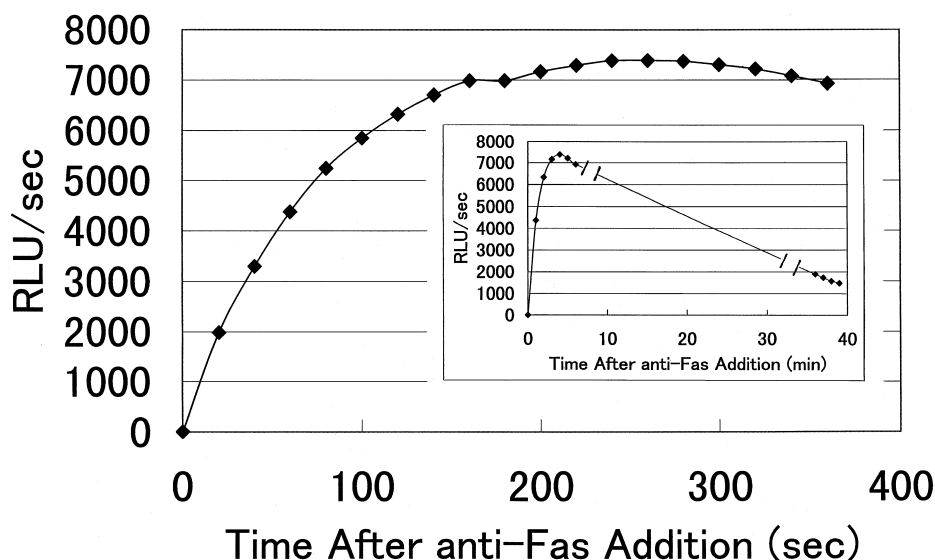


Fig. 1. Fas-mediated ROS generation. CL emission from BJAB cells stimulated with 1 µg/ml of anti-Fas mAb (clone CH-11) was monitored in a luminometer as described in Section 2. The data are representative of four separate experiments with the same results. Insert: CL emission from the stimulated cells was monitored at 1 min intervals for the initial 6 min and then again for 4 min from 30 min after the last measurement.

plate were cultured for 24 h in the presence or absence of the agents tested in a final volume of 0.1 ml. Next, 0.01 ml of the mixture of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) and 1-methoxy-5-methylphenazinium methylsulfate was added to each well. After incubation for 2–4 h, absorbance at 410 nm was measured in a multiplate reader using absorbance at 590 nm as a reference.

#### 2.4. DNA fragmentation assay

DNA fragmentation was determined by extraction of DNA followed by electrophoresis. Cells ( $10^6$  cells) were washed with PBS and lysed in a lysis buffer containing 10 mM Tris-HCl, pH 8.0, 0.5% SDS, 100 mM EDTA, 20 µg/ml of RNase, and 100 µg/ml of proteinase K, and incubated at 37°C for 1 h with gentle shaking. DNA was extracted with phenol saturated with 0.1 M Tris-HCl, pH 8.0 three times, once with phenol/chloroform (1:1 by volume), one with chloroform/isoamyl alcohol (1:24 by volume), and pelleted by 70% ethanol precipitation. The DNA was electrophoresed on a 2% agarose gel containing ethidium bromide and photographed under UV light.

#### 2.5. Measurement of CL

Luminol-dependent CL was measured with a luminometer (Bio-Lumat LB9507, Berthold, Wildbad, Germany). The assay mixture (0.25 ml) contained Hanks' balanced salt solution (HBSS) without phenol red, 8 mM luminol, and  $0.5\text{--}1 \times 10^6$  cells. Assay components were added to cuvettes, mixed, and placed immediately in the luminometer. Luminol alone caused a significant CL that returned to the background levels by 3 min. After 3 min preincubation, the reaction was started by the addition of the stimuli. At 20 s intervals up to 5–7 min the CL emission from the samples was recorded. The results are expressed as relative light units (RLU)/s where 1 RLU represents 10 photon counts.

#### 2.6. Flow cytometric analysis of surface antigens

For detection of surface antigen expression, cells ( $1 \times 10^6$ ) were suspended in phosphate-buffered saline (PBS) and incubated at 4°C for 60 min with the relevant mAbs and then incubated at 4°C for 30 min with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody. Finally, cells were extensively washed with PBS and analyzed with a flow cytometer (CytoAce 150, Jasco, Tokyo, Japan).

### 3. Results

Recently, it has been shown that several human B lympho-

ma cell lines such as BJAB, Ramos, and Raji express Fas on their surfaces and that they undergo cell death by apoptosis upon Fas stimulation [18]. Consistent with the previous report, when these B cells were treated with the agonistic anti-Fas mAb (clone CH-11), they underwent cell death as did Jurkat T cells, a typical Fas-sensitive cell line. When the genomic DNA from these cells was analyzed by agarose gel electrophoresis after the treatment, internucleosomal DNA fragmentation, a biochemical marker of apoptosis, was observed (not shown). These results indicate that all these cells die by apoptosis under the experimental conditions used here.

Next, we determined whether Fas ligation induced ROS generation in these Fas-sensitive cell types by monitoring CL, a strong indicator of ROS generation. Fas ligation induced a dramatic increase in CL emission in BJAB. The response could be first observed as rapidly as within 20 s, reaching a maximum at 5 min, and declining slowly thereafter (Fig. 1). A significant increase in ROS generation was observed for at least 40 min after the stimulation. Fas ligation induced ROS generation in a dose-dependent manner with a minimal effective dose of 200 ng/ml. The dose was comparable to that effective in inducing apoptosis. Fas ligation also induced ROS generation with similar kinetics in Ramos and Jurkat cells (not shown). We noticed considerable variation in the extent of Fas-mediated ROS generation, even when a single cell type was stimulated with the same amount of the mAb. On the other hand, Fas ligation by anti-Fas without apoptosis-inducing activity (clone ZB4) did not induce ROS generation in these cells. Engagement of a range of surface molecules on these B cells including surface IgM (sIgM), CD40, CD80, and CD81 did not induce cell death or ROS generation (not shown). These results demonstrate a good correlation between the ability to generate ROS and cell death.

To determine the possible involvement of cellular oxidases in Fas-mediated ROS generation, we tested the effect of DPI, a potent and specific inhibitor of flavonoid-containing enzymes such as NADPH oxidase and nitric oxide synthase (NOS) [19], on the response. Cells were incubated with the

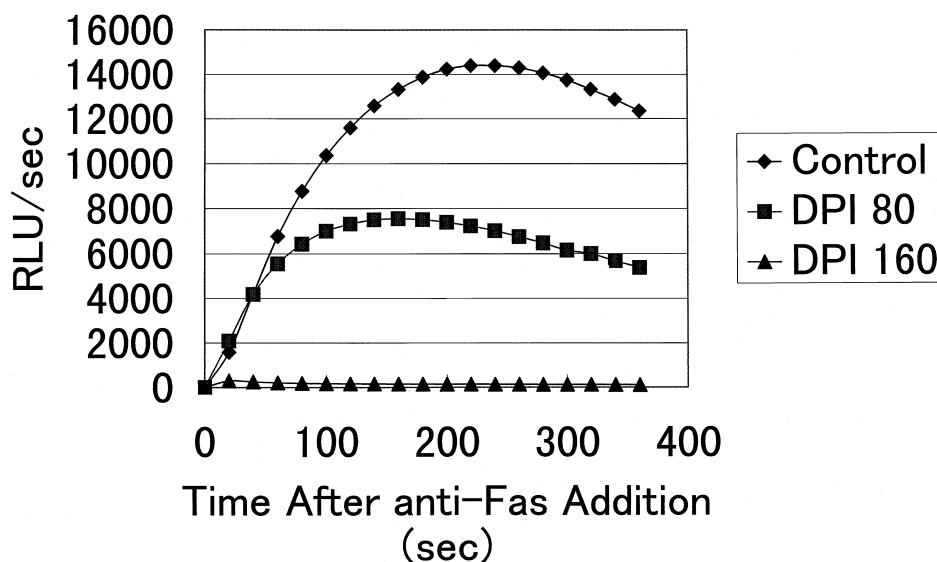


Fig. 2. Effect of DPI on Fas-mediated ROS generation. BJAB cells were incubated for 5 min with DPI at the concentrations indicated prior to stimulation with 1  $\mu$ g/ml of anti-Fas (clone CH-11). CL emission from the stimulated cells was monitored as described above. The data are representative of three separate experiments with the same results.

agent for 5 min and stimulated with the agonistic anti-Fas. DPI inhibited Fas-mediated ROS generation in a dose-dependent manner with a minimal effective dose of 80  $\mu$ M (Fig. 2). The agent (160  $\mu$ M) inhibited the generation completely. DPI at these concentrations showed no significant effect on cell behavior. In addition, when the cells tested were exposed to DPI for a longer period (30 min), 40  $\mu$ M DPI was enough to inhibit Fas-mediated ROS generation. Under these conditions, the final concentrations of the vehicle (DMSO) were as low as  $<0.1\%$  and showed a minimal effect on Fas-mediated ROS generation. A similar inhibitory effect of DPI on Fas-mediated ROS generation was observed in Jurkat cells (not shown).

#### 4. Discussion

Here we demonstrate that Fas ligation induces a rapid and sustained ROS generation in several Fas-sensitive cell types. In these cell types the kinetics of the generation was similar. The generation could be observed as rapidly as 20 s after the stimulation, reaching a maximum within 5 min, and slowly declining thereafter. A significant increase in the generation was observed at least by 40 min after Fas ligation. It has recently been shown that Fas ligation induces a rapid and transient increase in  $O_2^-$  synthesis [9]. The synthesis is rapid but transient; it can be observed within 0.3 min, peaking 1 min after stimulation and rapidly declining thereafter. After 3 min after stimulation, the synthesis is no longer observed. Thus, the ROS generation reported there was quite different from that observed here. Because the CL emission after Fas stimulation was not completely prevented by exogenous superoxide dismutase (30 U/ml), other radicals besides  $O_2^-$ , such as the hydroxyl radical ( $OH^\cdot$ ) might be involved in the reaction observed here.

Our results indicate that DPI inhibits Fas-mediated ROS generation. DPI is known to be a potent inhibitor of flavonoid-containing ROS-generating enzymes such as the NADPH oxidase and nitric oxide synthase (NOS) [19]. Be-

cause  $O_2^-$  seems to be produced after Fas ligation in our system, the inhibition of Fas-mediated ROS generation by DPI strongly suggests that the NADPH oxidase system is a source of ROS generation. NADPH oxidase is a multicomponent enzyme complex, present in the membranes of neutrophils and macrophages. Functional assembly of the oxidase catalyzes the transfer of one electron from cytosolic NADPH to molecular oxygen, generating superoxide. The key membrane-associated component of the NADPH oxidase is a heterodimeric flavocytochrome *b* which is composed of a 91-kDa glycoprotein (gp91<sup>phox</sup>) and a 22-kDa protein (p22<sup>phox</sup>) and is physically associated with two cytosolic proteins, p47<sup>phox</sup> and p67<sup>phox</sup>. Although the oxidase is known to play a critical role in host protection against infection, recent works show that virtually all cell types including B and T cells [20,21] have a similar oxidase system. Furthermore, in a variety of non-phagocytic cells, (NADPH-dependent)  $O_2^-/H_2O_2$  generation is observed in response to divergent extracellular stimuli such as IL-1 and TNF- $\alpha$  [22]. These observations strongly suggest that like the NADPH oxidase system in phagocytic cells, an NADPH oxidase-like system may function as a ROS-generating system in non-phagocytic cells. Our results suggest that at least in several cell types, stimulation via Fas activates such a NADPH-like oxidase system.

The significance of ROS generation in Fas-mediated apoptosis is at present unclear. However, our results indicate that the generation is correlated with apoptosis. In particular, anti-Fas without apoptosis-inducing activity failed to induce ROS generation, indicating that the generation is not merely the result of Fas ligation. The significance of ROS in Fas-mediated apoptosis in our system is supported by the fact that exogenous  $H_2O_2$  is a potent inducer of apoptosis in the cell lines used here. Recently, several laboratories including our own have shown that  $H_2O_2$  can stimulate protein tyrosine kinase (PTK) activation in lymphocytes [23,24]. In accordance with these observations, we have recently observed that Fas-mediated PTK activation is regulated by the cellular redox status and is prevented by DPI (Y. Suzuki et al., unpublished

results). In addition, PTK activation has been shown to be an early and requisite event in Fas-mediated apoptosis [25]. Taken together, these points raise the intriguing possibility that Fas-mediated ROS generation triggers a PTK-dependent biochemical cascade to apoptosis.

In conclusion, here we demonstrate rapid and specific ROS generation via NADPH oxidase activation during Fas-mediated apoptosis. Further investigations attempting to elucidate the significance of the generation in Fas-mediated apoptosis are ongoing.

## References

- [1] Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, M., Sameshima, S., Hase, A., Seto, Y. and Nagata, S. (1991) *Cell* 66, 233–243.
- [2] Nagata, S. and Golstein, P. (1995) *Science* 267, 1449–1455.
- [3] Daniel, P.T. and Klammer, P.H. (1994) *J. Immunol.* 152, 5624–5632.
- [4] Nagata, S. and Suda, T. (1995) *Immunol. Today* 16, 39–43.
- [5] Buttke, T.M. and Sandstrom, P.A. (1994) *Immunol. Today* 15, 7–10.
- [6] Matsuda, M., Masutani, H., Nakamura, H., Miyajima, S., Yamauchi, A., Yonehara, S., Uchida, A., Imajiri, K., Horiuchi, A. and Yodoi, J. (1991) *J. Immunol.* 147, 3837–3841.
- [7] Déas, O., Dumont, C., Mollereau, B., Métvier, D., Pasquier, C., Bernard-Pomier, G., Hirsch, F., Charpentier, B. and Senik, A. (1997) *Int. Immunol.* 9, 117–125.
- [8] Chiba, T., Takahashi, S., Sato, N., Ishii, S. and Kikuchi, K. (1996) *Eur. J. Immunol.* 26, 1164–1169.
- [9] Gulbins, E., Brenner, B., Schlottmann, K., Welsch, J., Heinle, H., Koppenhoefer, U., Linderkamp, O., Coggeshall, K.M. and Lang, F. (1996) *Immunology* 89, 205–212.
- [10] Um, H.-D., Orenstein, J.M. and Wahl, S.M. (1996) *J. Immunol.* 156, 3469–3477.
- [11] Dobbelsteen, D.J., Novel, C.S.I., Schlegel, J., Cotgreave, I.A., Orrenius, S. and Slater, F.G. (1996) *J. Biol. Chem.* 271, 15420–15427.
- [12] Dröge, W., Schulze-Osthoff, K., Mihm, S., Galter, D., Schenk, H., Eck, H.-P., Roth, S. and Gründer, H. (1994) *FASEB J.* 8, 1131–1138.
- [13] Bass, D.A., Parce, J.W., DeChatelet, L.R., Szejda, P., Seeds, M.C. and Thomas, M. (1983) *J. Immunol.* 130, 1910–1917.
- [14] Cathcart, R., Schwers, E. and Ames, B. (1983) *Anal. Biochem.* 134, 111–116.
- [15] Minkenberg, I. and Ferber, E. (1984) *J. Immunol. Methods* 71, 61–67.
- [16] Kobayashi, S., Imajoh-Ohmi, S., Kuribayashi, F., Nunoi, H., Nakamura, M. and Kanegasaki, S. (1995) *J. Biochem.* 117, 758–765.
- [17] Mosmann, T. (1983) *J. Immunol. Methods* 65, 55–63.
- [18] Kamitani, T., Nguyen, H.P. and Yeh, E.T.H. (1997) *J. Biol. Chem.* 272, 22307–22314.
- [19] Ellis, J.A., Mayer, S.J. and Jones, O.T.G. (1988) *Biochem. J.* 251, 887–891.
- [20] Volkman, D.J., Buescher, E.S., Gallin, J.L. and Fauci, A.S. (1984) *J. Immunol.* 133, 3006–3009.
- [21] Hancock, J.T., Maly, F.-E. and Jones, O.T.G. (1989) *Biochem. J.* 262, 373–375.
- [22] Meier, B., Radeke, H.H., Selle, S., Younes, M., Sies, H., Resch, K. and Habermehl, G.G. (1989) *Biochem. J.* 263, 539–545.
- [23] Schieven, G.L., Kirihara, J.M., Burg, D.L., Geahlen, R.L. and Ledbetter, J.A. (1993) *J. Biol. Chem.* 268, 16688–16692.
- [24] Suzuki, Y., Ohsugi, K. and Ono, Y. (1996) *Immunology* 87, 396–401.
- [25] Eischen, C.M., Dick, C.J. and Leibson, P.J. (1994) *J. Immunol.* 153, 1947–1953.